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Susceptibility of rabbits to *Treponema pallidum* after infection with *Mycobacterium bovis*

S. GRAVES

From the Department of Microbiology, Monash University Medical School, Alfred Hospital, Prahran, Australia

SUMMARY Rabbits stimulated with *Mycobacterium bovis* (strain BCG) one month before challenge with *Treponema pallidum* (Nichols) did not show any modification in their development of syphilitic lesions. A second infection with BCG given at the same time and at the same intradermal site as the *T. pallidum* challenge also failed to protect the rabbits against syphilis. Thus the non-specific activation of cell-mediated immunity by BCG does not appear to protect rabbits against *T. pallidum* infection even when both activation and challenge take place in the dermis. The role of the macrophage in syphilis remains obscure.

Introduction

Human syphilis and the animal model disease (*Treponema pallidum* infection in rabbits) results in both a humoral and a cell-mediated immune response by the host (Kiraly, 1976; Musher *et al.*, 1976), but the relative contribution of each to overall immunity has yet to be determined. Antibody alone is not fully protective, since in passive transfer experiments rabbits ultimately succumb to infection (Perine *et al.*, 1973; Sepetjian *et al.*, 1973; Turner *et al.*, 1973; Graves and Johnson, 1975; Bishop and Miller, 1976; Weiser *et al.*, 1976). This suggests that cell-mediated immune responses also play a part in the establishment of the immune state. There is evidence both for and against this (Levene *et al.*, 1971; Metzger and Smogor, 1975; Schell and Musher, 1975; Schell *et al.*, 1975a, b; Wicker and Wicker, 1975; Baughn *et al.*, 1977b; Metzger *et al.*, 1977; Wicker and Wicker, 1977).

Although activation of host macrophages is one of the main effector mechanisms of cell-mediated immunity, resulting in enhanced intra-macrophage killing of phagocytosed micro-organisms, it has been shown that the non-specific activation of rabbit macrophages by living *Mycobacterium bovis* (strain BCG) or *Propionibacterium acnes* did not render rabbits more resistant to a *T. pallidum* challenge (Graves and Johnson, 1975; Schell *et al.*, 1975b; Baughn *et al.*, 1977a).

The experiment reported here was devised to stimulate host immunity with BCG at the same site as the *T. pallidum* infection, the dermis. It was thought that the activation of the rabbit macrophages in the dermis by injection of *Mycobacterium tuberculosis* (BCG strain) at that site would be more protective than the previously used technique of intravenous injection of BCG (Graves and Johnson, 1975), resulting mainly in activated macrophages in liver and spleen. This is because the *T. pallidum* challenge is normally given intradermally into the shaved back of the rabbit. Lukehart and Miller (1978) have previously commented on the need to activate macrophages at the actual site of *T. pallidum* challenge before drawing conclusions about their function in this disease. They have demonstrated macrophage uptake of *T. pallidum in vitro*.

As reported in this paper, rabbits stimulated with BCG were still infected with *T. pallidum* and no protection was evident, suggesting that activated macrophages do not play a significant part in resistance to reinfection in rabbit syphilis. Baughn *et al.* (1977a) came to a similar conclusion after intradermally injected *Propionibacterium acnes* failed to protect rabbits against an intradermal challenge with *T. pallidum*.

Materials and methods

All rabbit groups (A, B, C, D, and E) contained three rabbits (Table).

Address for reprints: Dr S. Graves, Department of Microbiology, Monash University Medical School, Alfred Hospital, Prahran, Victoria 3181, Australia

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Table *Inoculation of five groups of rabbits with Mycobacterium bovis (BCG) and Treponema pallidum*

	Inoculation time			
	Zero	*One month**		*Six months*
Experimental groups	*BCG (2 mg i.v.)*	*BCG†*	*T. pallidum‡*	*T. pallidum§*
A	Yes	Yes	Yes	Yes
B	Yes	No	Yes	Yes
C	No	Yes	Yes	Yes
D	No	No	Yes	Yes
E	No	No	No	Yes

*In groups A and C the BCG and *T. pallidum* challenge was given simultaneously in the same syringe after being mixed within five minutes of inoculation.
†Eight sites were inoculated; 250 μg/site, total 2 mg.
‡Eight sites were inoculated; two each with 10^4, 10^3, 10^2, and 10 intradermally.
§Eight sites were inoculated; two each with 10^6, 10^5, 10^4, and 10^3 intradermally.

INFECTION WITH BCG

At zero time

Rabbits in groups A and B (Table) were pretreated with 2 mg BCG (dry weight) given intravenously in saline one month before the first challenge with *T. pallidum*. BCG was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia.

At one month

Rabbits in groups A and C (Table) were infected with BCG at the same time as the first challenge with *T. pallidum* (see below). The BCG was inoculated into eight intradermal sites on the shaved back of each rabbit; 250 μg BCG (dry weight) was inoculated per site, a total of 2 mg per rabbit.

CHALLENGE WITH *T. PALLIDUM*

At one month

All rabbit groups, except E (Table), were challenged at eight intradermal shaved sites per rabbit with duplicate doses of 10^4, 10^3, 10^2, and 10 *T. pallidum* (see below for preparation). The BCG and *T. pallidum* were independently suspended in anaerobic bacterial medium (Graves *et al.*, 1975) and mixed together immediately before inoculation into the rabbit using the same syringe.

At six months

All rabbit groups (Table) were challenged at eight intradermal shaved sites per rabbit with duplicate doses of 10^6, 10^5, 10^4, and 10^3 *T. pallidum*.

MONITORING THE COURSE OF INFECTION

After the first challenge with *T. pallidum* rabbits were examined daily for one month, every two or three days thereafter for another month, and every week thereafter for a further 2½ months, a total observation period of 4½ months. After the challenge with *T. pallidum* at six months, rabbits were observed for a total of 2 months, although no further lesions appeared after the first 3½ weeks. The rabbits were shaved regularly and the degree of induration was recorded. The appearance of any secondary syphilitic lesions was noted. The experimental rabbits were individually housed at 16-19°C and fed antibiotic-free rabbit pellets and water in unrestricted quantities. Only male rabbits were used.

PREPARATION OF CHALLENGE *T. PALLIDUM*

T. pallidum was propagated in the testes of male rabbits by inoculating 5×10^7 viable *T. pallidum* per testis and harvesting it approximately 11 days later when a well developed orchitis was present. Elution of the *T. pallidum* from the minced orchitic testis required several sequential five-minute washings with 5-10 ml of sterile, prereduced anaerobic medium (Graves *et al.*, 1975). The washings were pooled under a flow of sterile oxygen-free nitrogen, counts were made in a Petroff-Hauser chamber, and the suspension was diluted to the required concentration for rabbit challenge.

SEROLOGICAL TESTING OF INFECTED RABBITS

To test for anti-cardiolipin (reaginic) antibody the rapid plasma reagin (RPR) card test (Hynson, Wescott and Dunning, Baltimore) was used qualitatively. To test for anti-treponemal antibody the *T. pallidum* haemagglutination (TPHA) test (Fujizoki Pharmaceutical Co. Ltd., Shinjuku-ku, Tokyo) was used quantitatively. In the latter test titres of 1/80 or greater were considered to indicate antigenic stimulation with *T. pallidum*.

Results

SINGLE INFECTION WITH BCG (GROUPS B AND C)

At zero time

When rabbits in group B were challenged with *T. pallidum* (at one month) dermal syphilitic lesions appeared after incubation periods which were almost identical to those of control rabbits (group D). At the 10^4 challenge sites lesions appeared after 14 days (control 13 days); at the 10^3 challenge sites after 15 days (control 17 days); at the 10^2 challenge sites after 17 days (control 18 days); and at the 10 challenge site after 19 days (control 18 days). These incubation periods represent the mean of six lesions (two per rabbit for each of three rabbits). Thus BCG infection at zero time did not modify the incubation period of the subsequent syphilitic infection indicating that it

did not retard the growth of *T. pallidum* in these rabbits.

At one month

Rabbits in group C received their BCG infection at the same time and same site as their first challenge with *T. pallidum*. They did not receive BCG infection at zero time. Three days after infection they developed indurations of approximately 1 cm diameter at the sites of infection. These lesions faded after three to four weeks and disappeared by five weeks. All eight inoculation sites per rabbit showed similar indurated lesions, which appeared and disappeared together, unlike the control rabbits (group D) in which the sequential appearance of syphilitic lesions paralleled the size of the *T. pallidum* inoculum at that site. For this reason the lesions seemed to be due primarily to the growth of BCG, since each inoculation site received the same dose of BCG (250 μg). In two of the three rabbits in group C, the indurated lesions, after disappearing, reappeared again in a milder form at the original sites of inoculation about two months after the initial infection. They persisted for another two to four weeks before again disappearing. This further suggested that the indurations were due to BCG infection because *T. pallidum* lesions never reappear at the same site once they have regressed. Apparently the BCG growth masked any *T. pallidum* lesion development. The *T. pallidum* was not destroyed, however, since the rabbits subsequently developed syphilitic immunity. Furthermore, two of the three rabbits in group C did develop secondary, dermal, syphilitic lesions at sites on the shaved backs of the rabbits other than those inoculated. These appeared between day 36 and day 40 after inoculation, which is the normal time for secondary lesions to appear in *T. pallidum*-infected (but otherwise untreated) rabbits that do develop secondary lesions. The appearance of secondary lesions usually occurs in only a few rabbits after intradermal inoculation (Chesney and Schipper, 1950; Turner and Hollander, 1957).

DOUBLE INFECTION WITH BCG (GROUP A)

Rabbits in group A received two infections with BCG, one at zero time and another one month later. One to two days after the BCG infection at one month (and associated *T. pallidum* challenge) all eight inoculation sites showed strong delayed type hypersensitivity (DHS) reactions of equal intensity. However the induration did not fade, as would be the case with normal DHS, but remained strong (greater than 1 cm diameter) until about one month after infection. No sign of the classical syphilitic lesion (neither primary nor secondary) appeared on these rabbits, and, as before, the lesion seemed to be due primarily to BCG growth or host hypersensitivity to BCG or both. Thus, *T. pallidum* growth was masked but evidently did occur because the rabbits subsequently developed syphilitic immunity.

CHALLENGE WITH *T. PALLIDUM* AT SIX MONTHS

After a second infection with *T. pallidum* at six months, all the rabbits of group A, B, C, and D failed to develop any syphilitic lesions whereas the control rabbits of group E all developed lesions with the following average incubation times: five days (10^6 inoculum); eight days (10^5 inoculum); 14 days (10^4 inoculum), and 21 days (10^3 inoculum). This demonstrates that the initial *T. pallidum* inoculation in all groups, including groups A and C where no primary syphilitic lesions were observed, did result in infection as shown by the subsequent development of immunity to repeat challenge.

SEROLOGICAL RESPONSES

Anti-cardiolipin antibody was synthesised by all rabbits in groups A, B, C, and D between one and two months after infection. Anti-treponemal antibodies (TPHA test) were also synthesised by all the above rabbits by two months after infection with *T. pallidum*. Titres did not vary significantly from one group to another.

Discussion

Mycobacterial species, especially BCG, and chemical fractions derived from mycobacteria have been widely used to enhance non-specific cell-mediated immunity to a variety of micro-organisms and tumours. In the case of bacteria it acts by antigen-induced blast transformation of specific T-lymphocytes and the release of lymphokines which activate normal host macrophages to develop enhanced intracellular killing ability. This latter ability is mainly non-specific (Mackaness, 1971).

Earlier work by Graves and Johnson (1975) demonstrated that BCG given intravenously one month before intradermal challenge with *T. pallidum* failed to modify the development of syphilitic lesions in the rabbit. The simultaneous administration of immune syphilitic serum markedly delayed the onset of lesions but there was no synergy evident between the immune serum and the BCG, suggesting that opsonisation had not occurred. Schell *et al.* (1975b) also reported that BCG failed to suppress a challenge with intravenous *T. pallidum* in rabbits while Baughn *et al.* (1977a) noted that the use of *Propionibacterium acnes* as a macrophage stimulant failed to modify the course of an infection with *T. pallidum* in rabbits even when given intradermally. Harris and

Thoen (1977) reported that the intradermal injection of BCG in mineral oil did protect rabbits from challenge with *T. pallidum* five days later. This finding is not consistent with the present observations, although differences in experimental procedure did occur.

There is evidence that cell-mediated immunity does develop in the host (rabbit or man) during the course of an infection with *T. pallidum* (Musher *et al.*, 1976; Kiraly, 1976), and possibly it plays some role in the regression of lesions and the ultimate recovery of the host—for example, in human infections where recovery is sometimes spontaneous. Is phagocytosis by macrophages an important effector mechanism of cell-mediated immunity in syphilis? After *in-vitro* studies, Lukehart and Miller (1978) claim that it is and that immune serum significantly enhanced *in-vitro* phagocytosis of *T. pallidum* by rabbit peritoneal macrophages. If macrophages are important, why does BCG not enhance *T. pallidum* killing? Perhaps the activation of *T. pallidum*-killing macrophages is highly specific, requiring certain obligatory treponemal antigens, and cannot therefore be induced with BCG.

Alternatively, the macrophage may not play an important role in immunity. *T. pallidum* could be killed by some other mechanism, such as immune cytolysis involving specific T-lymphocytes or antibody-mediated K-cell killing. Electron microscopical observations of early syphilitic lesions have shown that very few *T. pallidum* are intracellular and that most are extracellular (Azar *et al.*, 1970; Lauderdale and Goldman, 1972). This, of course, does not rule out a role for phagocytic cells later in the infection (during the immune stage) but it does suggest that rapid and efficient uptake of *T. pallidum* by normal host cells does not occur as part of the initial response to infection. Assuming that the *T. pallidum* cell is not susceptible to phagocytosis in the normal host, it is perhaps not surprising that enhancing the intracellular killing power of the macrophages with BCG does not result in enhanced killing of *T. pallidum*. Wicker *et al.* (1977) reported that leucocytes (monocytes and polymorphonuclear leucocytes) from syphilitic rabbits showed an increased incidence of nitroblue tetrazolium (NBT) reduction. This increase was observed 10 days after infection and reached a maximum 30 days after infection with *T. pallidum*; the NBT-positive leucocytes returned to normal 50 days after infection. This corresponds to the period taken for the lesion to heal but not to the period of maximum immunity to reinfection. Rabbits are not usually fully resistant to *T. pallidum* challenge until about three months after infection (Turner and Hollander, 1957). Does this suggest that phagocytosis and induction of immunity are temporally well separated? It is the immunologically more important macrophages that are the key to this question and not the polymorphonuclear leucocytes. Although human monocytes did not phagocytose virulent *T. pallidum in vitro* (Brause and Roberts, 1978), the role of the macrophage in the syphilitic lesion is still to be determined.

The inability of spleen cells from immune syphilitic rabbits to confer immunity to challenge (Baughn *et al.*, 1977b), even with inbred recipient rabbits, implies that lymphocytes (of either subclass T or B) are not effector cells in the immune animal. Antibodies are the best documented line of defence against *T. pallidum* in rabbits, possibly acting in concert with some cellular elements of the host, since alone they do ameliorate infections but without giving complete protection. Metzger and Smogor (1975) reported some degree of immunity following passive transfer of lymphocytes from immune rabbit lymphnodes, but this would have included B (antibody-producing) lymphocytes, so the effect observed may have been due to antibody synthesis in the recipient rabbit.

The present study has shown the inability of BCG, a commonly used reticuloendothelial system stimulant, to stimulate the rabbit's immune system against *T. pallidum*. The injection of BCG into the same intradermal sites as the *T. pallidum* did mask the development of syphilitic lesions due to the growth of, or hypersensitivity to, BCG or both. The normal method of determining if these rabbits were infected with *T. pallidum*, in the absence of observable, characteristic lesions, is to wait until several months after inoculation and then to transfer excised lymph nodes to the testes of a normal rabbit. This latter rabbit is then examined regularly for a syphilitic orchitis in which *T. pallidum* is demonstrable by darkfield microscopy (Miller, 1971). This method was not used in this study owing to a shortage of space in our laboratory and because an equally sound technique was available that did not require additional rabbits. This method is based on the observation that after an untreated syphilitic infection of about three months' duration these rabbits are refractory to challenge with the same strain of *T. pallidum*. Hence, when no lesions are observed a rabbit can be tested for the presence of infection by challenging with *T. pallidum* at least three months after the time of suspected infection. In this experiment a time period of five months was used to be certain that immunity had developed in the infected rabbits. If, on challenge, no lesions develop the rabbits can be assumed to have been previously infected, thus explaining their current state of immunity. If, on the other hand, lesions did occur on challenge the implication is that the

previous exposure to *T. pallidum* had not resulted in a syphilitic infection.

In the present study, all rabbits were shown to have been infected by the challenge with *T. pallidum* at one month by virtue of their immunity to the challenge with *T. pallidum* at six months. In support of this conclusion, all rabbits developed antibodies to cardiolipin (reagins) and *T. pallidum* antigens as shown by the RPR and the TPHA tests respectively. Although a serological response, as measured by the TPHA test, could have developed by simple antigenic stimulation and without an infection the development of cardiolipin antibodies represents tissue destruction caused by *T. pallidum* infection (Fredericksson *et al.*, 1968).

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